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Substrate-induced lipase gene expression and aflatoxin production in *Aspergillus parasiticus* and *Aspergillus flavus*

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ABSTRACT

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Aims: To establish a relationship between lipase gene expression and aflatoxin production by cloning the *lipA* gene and studying its expression pattern in several aflatoxigenic and nontoxigenic isolates of *Aspergillus flavus* and *A. parasiticus*.

Methods and Results: We have cloned a gene, *lipA*, that encodes a lipase involved in the breakdown of lipids from aflatoxin-producing *A. flavus*, *A. parasiticus* and two nonaflatoxigenic *A. flavus* isolates, wool-1 and wool-2. The *lipA* gene was transcribed under diverse media conditions, however, no mature mRNA was detected unless the growth medium was supplemented with 0·5% soya bean or peanut oil or the fungus was grown in lipid-rich medium such as coconut medium. The expression of the lipase gene (mature mRNA) under substrate-induced conditions correlated well with aflatoxin production in aflatoxigenic species *A. flavus* (SRRC 1007) and *A. parasiticus* (SRRC 143).

Conclusions: Substrate-induced lipase gene expression might be indirectly related to aflatoxin formation by providing the basic building block 'acetate' for aflatoxin synthesis. No direct relationship between lipid metabolism and aflatoxin production can be ascertained, however, lipase gene expression correlates well with aflatoxin formation.

Significance and Impact of the Study: Lipid substrate induces and promotes aflatoxin formation. It gives insight into genetic and biochemical aspects of aflatoxin formation.

Keywords: aflatoxin, *Aspergillus flavus*, *Aspergillus parasiticus*, lipase, lipid-induction, nutritional regulation.

INTRODUCTION

Aflatoxins, produced predominantly by fungi such as *Aspergillus flavus* and *A. parasiticus*, are among the most potent natural carcinogens known. Contamination of agricultural commodities with aflatoxins are of serious health and economic concern worldwide (Jelinek *et al.* 1989). Tremendous progress has been recently made in aflatoxin research. In turn the discovery of the aflatoxin pathway gene cluster (Trail *et al.* 1995; Yu *et al.* 1995; Brown *et al.* 1996) led to a rapid identification of the majority of genes involved in aflatoxin biosynthesis (Yabe *et al.* 1989, 1998; Bhatnagar *et al.* 1992, 2002; Cleveland and Bhatnagar 1992; Chang *et al.* 1993, 1995; Payne *et al.* 1993; Yu *et al.* 1993, 1995, 1998, 2000a, b; Trail *et al.* 1995; Cleveland *et al.* 1997; Woloshuk *et al.* 1997; Payne and Brown 1998; Woloshuk and Prieto 1998).

Aflatoxin production is known to be influenced by many biotic and abiotic environmental factors such as temperature, water stress, pH, nutritional conditions (Adye and Mateles 1964; Dutton 1988; Payne and Brown 1998; Chang *et al.* 2000), and interaction between host and invading fungi. It is known that carbon and nitrogen sources play a vital role in the regulation of aflatoxin production (Adye and Mateles 1964; Dutton 1988; Payne and Brown 1998; Chang *et al.* 2000; Yu *et al.* 2000c; Aziz *et al.* 2002). Simple sugars

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such as glucose, sucrose, maltose and galactose induce aflatoxin production, while complex carbohydrates such as starch and peptone do not (Adye and Mateles 1964; Payne and Brown 1998). Glucose or sucrose has the same effect on inducing aflatoxin formation.

Aflatoxin biosynthesis is a complex process. The aflatoxin pathway gene cluster within a 80-kb DNA region in *A. flavus* and *A. parasiticus* (Yu *et al.* 1995; Cleveland *et al.* 1997) are under the tight control of a positive regulatory gene *aflR* (Chang *et al.* 1993, 1995; Payne *et al.* 1993; Ehrlich *et al.* 1999) and *aflJ* (Meyers *et al.* 1998; Chang and Yu 2002). The genetic regulation of aflatoxin production in response to environmental and nutritional factors however remains poorly understood as is the regulation and interaction of the *aflR* gene with other pathway regulators or signals (Chang *et al.* 1995; Payne and Brown 1998).

Although the relationship between lipids and aflatoxin production (Fanelli *et al.* 1983; Fanelli and Fabbri 1989; De Luca *et al.* 1995; Fanelli *et al.* 1995; Aziz *et al.* 2002) and lipase activity in nonaflatoxigenic fungi (Ohnishi 1982a, b; Yamaguchi *et al.* 1991; Tsuchiya *et al.* 1996) have been reported, no expression of lipase genes in aflatoxigenic fungi was reported. The effects of lipids on fungal growth and aflatoxin production have been studied in *A. flavus* and *A. parasiticus* by many researchers (Fanelli *et al.* 1983, 1995; Fanelli and Fabbri 1989; De Luca *et al.* 1995) who demonstrated that lipophilic epoxy fatty acids stimulated aflatoxin production in toxigenic fungi (Fanelli *et al.* 1983). Ergosterol oxidation was also found to induce both fungal growth and aflatoxin production (De Luca *et al.* 1995). Saturated free fatty acids support fungal growth and aflatoxin production, while the unsaturated free fatty acids inhibit fungal growth (Fanelli and Fabbri 1989). Lipoperoxidation of unsaturated free fatty acids produces lipoperoxides, which are presumed to inhibit fungal growth and reduce aflatoxin biosynthesis (Fanelli and Fabbri 1989).

Lipases are lipolytic enzymes produced by nonaflatoxigenic fungi closely related to *A. flavus* such as *A. oryzae* (Ohnishi 1982a, b; Tsuchiya *et al.* 1996) and other fungi not related to *A. flavus* such as *Penicillium camembertii* (Yamaguchi *et al.* 1991) which by hydrolysis of triglycerides modify fungal lipid composition. In order to establish the linkage between lipid metabolism and aflatoxin production at a molecular level, we have cloned a gene, *lipA*, encoding a lipase from aflatoxigenic strains *A. parasiticus* SRRC 143 and *A. flavus* SRRC 1007. In addition, *lipA* from two nonaflatoxigenic *A. flavus* isolates, wool-1 and wool-2, demonstrating the ability to degrade wool possibly by proteases and lipases, was also cloned in this study. The expression patterns of *lipA* in the two aflatoxigenic species, the two nonafltoxigenic *A. flavus* isolates, and an *A. parasiticus* nonaflatoxigenic *aflR*-disrupted mutant CS10N2-disA (Takahashi *et al.* 2002) were studied under aflatoxin-inducive and noninducive medium conditions.

MATERIALS AND METHODS

Fungal strains and culture conditions

Fungal strains used in this study were the following: (i) *A. parasiticus* SRRC 143 (ATCC 56775) which produces aflatoxins B_1, B_2, G_1 and G_2; (ii) *A. flavus* SRRC 1007 which produces aflatoxins B_1 and B_2; (iii) two nonaflatoxigenic *A. flavus* isolates, wool-1 and wool-2, respectively, which do not produce any aflatoxin or aflatoxin intermediates and are capable of degrading wool; and (iv) an *aflR*-disrupted mutant strain of *A. parasiticus* SRRC 143, CS10N2-disA, in which the aflatoxin pathway positive regulatory gene, *aflR*, was disrupted. As a result, no aflatoxin pathway genes are expressed and no aflatoxin is produced (in this *aflR*-disabled mutant). Fungal strains were maintained on potato dextrose agar plates (Difco Laboratories, Detroit, MI, USA). For conidial production, spores were inoculated on Petri dishes (100 × 15 mm style; Falcon, Becton Dickinson and Company, Franklin Lakes, NJ, USA) containing 20 ml of V8 agar solid medium [50 ml l^{-1} V8 juice, a commercial beverage (Campbell Soup Company, Camden, NJ, USA, http://www.v8juice.com] consisting of eight vegetable juices, 20 g l^{-1} agar, pH 5·2). The cultures were incubated at 30°C temperature. For detection of gene expression and aflatoxin production, fungal mycelia were grown as submerged cultures in aflatoxin-inducive GMS medium [containing (g l^{-1}): sucrose, 50; ammonium sulphate, 3; potassium phosphate, 10; magnesium sulphate, 2; micronutrient mixture; 1 ml, pH 4·5] (Adye and Mateles 1964) and nonaflatoxin-inducive peptone mineral salt (PMS) medium in which sucrose was replaced with an equal amount of peptone (Adye and Mateles 1964). For detection of *lipA* gene expression under lipid-induced conditions, the sucrose mineral salt (GMS) and PMS media were supplemented with 0·5% soya bean or peanut oil. The coconut medium (CAM) was reported to support aflatoxin production (Davis *et al.* 1987; Dyer and McCammon 1994) like sucrose or glucose. Coconut medium is a lipid-rich medium and was also used to induce *lipA* gene expression. The CAM was made by blending 200 g of commercially sold solid coconut with 400 ml hot distilled water. The soluble coconut nutrients were retained after filtering out undissolved material by passage through Miracloth, the resulting liquid filtrate was adjusted to pH 7·0 using 3 M NaOH, and after sterilization was used to support submerged fungal growth. Growth media for the *aflR*-disrupted mutant (CS10N2-disA) was supplemented with uracil at a final concentration of 0·1 mg ml^{-1} as the *pyrG* gene was also disrupted to create

marker selection system for fungal transformation in this mutant. All liquid cultures were grown for 72 h at 30°C with constant shaking at 150 rev min^{-1}.

Cloning and DNA sequencing

The lipase gene, *lipA*, was cloned from *A. parasiticus* SRRC 143 and *A. flavus* SRRC 1007 by polymerase chain reaction (PCR) using primers designed based on a homologue of *lipA* in *A. oryzae*, *mdlB* (accession no. D85895). The primers used for *lipA* gene cloning are, forward primer (lipF2): 5′-CCAAGCTTTGCAACCAAGCCTGTCG-3′ and reverse primer (lipR2): 5′-CTGCAGGTGTAGTGTGCTTGG-CCGA-3′. The corresponding cDNA sequences were also cloned by reverse transcriptase PCR (RT-PCR) using primers designed at the putative translation start and stop sites. The primers are, forward primer (lipF1): 5′-GAT-GCGCTTCCTCTCCGGCTTCG-3′ and reverse primer (lipF1): 5′-TTAGCGGAAGGGCAATCCATGAC-3′. The genomic and cDNA sequences of *lipA* gene were determined by direct sequencing of the PCR products. The two pairs of primers were also used for genomic DNA (PCR products) and cDNA (RT-PCR products) sequencing as sequencing primers. All of the primers used in this study were made with a DNA synthesizer (model 380A; Applied Biosystems, Foster City, CA, USA).

RNA purification and RT-PCR experiments

All of the total RNAs were isolated from 72 h mycelia of the fungal strains in respective liquid media (GMS and PMS) using RNeasy Total RNA Kit (Qiagen Inc., Valencia, CA, USA) from the mycelia. The mRNA was purified subsequently using PolyATract mRNA isolation System (Promega Corp., Madison, WI, USA). First strand cDNA was synthesized using Advantage RT-for-PCR Kit (Clontech, Palo Alto, CA, USA). The first strand cDNA was used as the template in RT-PCR experiment to detect *lipA* mRNAs under different medium conditions. RT-PCR was performed with denaturing at 94°C for 15 s, annealing at 56°C for 30 s, extension at 72°C for 2 min, 30 cycles with a final extension at 72°C for 5 min. PCR products were separated in 1% agarose gel under 80 V for 90 min.

Aflatoxin extraction and thin-layer chromatography

Aflatoxins were extracted from the fungal mycelia grown in different liquid media after 72 h of growth at 30°C with an acetone/chloroform solvent system (1 : 1 ratio, v/v; Dutton 1988). The extracted aflatoxin metabolites were assayed by loading 3 μl of a 300-μl sample onto thin-layer chromatography (TLC) plates (catalog no. 7001-04, silica gel; J. T. Baker, Inc., Phillipsburg, NJ, USA) and subsequent separation achieved with an ether : methanol : water (96 : 3 : 1, v/v/v) solvent system. The solvent separated aflatoxins were visualized under 365 nm long wave ultraviolet and the amount of aflatoxins was quantified visually by comparing the brightness of the spots with a series of standard samples of known concentration by densitometry. The aflatoxin standards were purchased from Sigma Co. (St Louis, MO, USA) and replicate analyses were performed.

GENBANK ACCESSION NUMBERS

The *lipA* gene sequence data from *A. parasiticus* SRRC 143 and from *A. flavus* SRRC 1007 have been submitted to GenBank databases under accession numbers AF404488 and AF404489, respectively (http://www.ncbi.nlm.nih.gov).

RESULTS

The *lipA* gene encodes a polypeptide consisting of 306 amino acid residues

The *lipA* gene from *A. parasiticus* SRRC 143 and *A. flavus* SRRC 1007 was cloned by PCR using primers based on a homologue of *lipA* in *A. oryzae*, *mdlB* (Tsuchiya *et al.* 1996). The genomic DNA sequences from *A. parasiticus* and *A. flavus* were found to be 1654 and 1665 bp, respectively. The corresponding cDNA sequences were also cloned by RT-PCR using primers designed at the putative translation start and stop sites. Two intervening sequences of 52 bp each were found near the N-terminal region. The coding region consists of 921 bp, capable of encoding 306 amino acids (AA) (Fig. 1) with a calculated molecular mass of 33·5 kDa. There were 27 nucleotide substitutions and deletions within the coding region of the lipase from *A. flavus* compared with that of *A. parasiticus*. However, only five of them resulted in changes of AA residues (Fig. 1). The genomic DNA sequences of *A. flavus lipA* and *A. oryzae mdlB* are identical (Tsuchiya *et al.* 1996) (Fig. 1) probably because these fungal species are highly related. The homology of the AA sequences encoded by *lipA* between *A. parasiticus* and *A. flavus* (*A. oryzae*) is 98·7%. However, a relatively significant degree of homology (62·7%) at the AA level was also found between *A. parasiticus* and a nonrelated fungal species *P. camembertii* (Yamaguchi *et al.* 1991; Fig. 1).

Expression of *lipA* gene under lipid-induced condition

The functional expressions of the *lipA* gene of these *Aspergillus* species under several nutritional conditions were studied by RT-PCR experiments. When *A. parasiticus* and *A. flavus* mycelia were grown in aflatoxin-inducive (GMS)

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A.p.lipA 001 MRFLSGFVSVLSSVALLGYAYPTAIDVRDIPTTQLEDFKFWVQYAAATYC
A.f.lipA     ------------------------------------------------
A.o.mdlB     ------------------------------------------------
P.c.mdlA     --.--F-TA.--A--S----L-GKLQS--VS-SE-DQ-E--------S-Y

A.p.lipA 051 PNNYVAKDGEKLNCSVGNCPDVEAADSTVKLSFSDDTITDTAGFVAVDNT
A.f.lipA     -------------------------G------------------------
A.o.mdlB     -------------------------G------------------------
P.c.mdlA     EAD-T-QV-D--S--K----E---TGA--SYD---S-------YI---H-

A.p.lipA 101 NKAIVVAFRGSYSIRNWVTDATFPQTDPGLCDGCKAELGFWTAWKVVRDR
A.f.lipA     ------------------------------------------------
A.o.mdlB     ------------------------------------------------
P.c.mdlA     -S-V-L-------V----A----VH-N-------L------SS--L---D

A.p.lipA 151 IIKTLDELKPEHSDYKIVVVGHSLGAAIASLAAADLRTKNYD.AILYAYA
A.f.lipA     ----------------------------------------.-------
A.o.mdlB     ----------------------------------------.-------
P.c.mdlA     ---E-K-VVAQNPN-EL---GHSLG--V-T---T---G-G-PS-K-----

A.p.lipA 200 APRVANKPLAEFITNQGNNYRFTHNDDPVPKLPLLTMGYVHISPEYYITA
A.f.lipA     ------------------------------------------------
A.o.mdlB     ------------------------------------------------
P.c.mdlA     S---G-AA--KY--A----F----TN---------S-----V----W--S

A.p.lipA 250 PDNTTVTDNQVTVLGGYVNFEGNTGTSGGLPDLLAFHSHVWYFIHADACK
A.f.lipA     --------------D-----K------------------------------
A.o.mdlB     --------------D-----K------------------------------
P.c.mdlA     -N-A--STSDIK-ID-D-S-D-----..---L-TD-EA-I---VQV--G-

A.p.lipA 300 GHGLPFR*
A.f.lipA     -P---L-*
A.o.mdlB     -P---L-*
P.c.mdlA     -P----KRV*
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Fig. 1 Comparisons of the lipase amino acid sequences from *Aspergillus parasiticus*, *A. flavus*, *A. oryzae* and *Penicillium camembertii*. *A.p.*, *A.f.*, *A.o.* and *P.c.* are abbreviations for sequences from *A. parasiticus* SRRC 143, *A. flavus* SRRC 1007, *A. oryzae*, and *P. camembertii*, respectively. The numbers to the left indicate the amino acid (AA) sequence starting from the translation start methionine (M). Identical AA are represented by a dash (–) and a gap is represented by a dot (.). The translation stop codon is represented by a star (*). The first AA residue of putative mature enzyme (Tsuchiya *et al.* 1996) at position 29 (D) is printed in boldface letter and underlined. The conserved AA sequence motif for lipolytic enzymes (GXSXG) is underlined and the conserved AA residues are printed in boldface letters. The serine (S), aspartic acid (D) and histidine (H), that form a catalytic triad, are printed in boldface letters and double underlined. The putative N-glycosylation site (Tsuchiya *et al.* 1996) at position 252 is also underlined

and nonaflatoxin-inducive (PMS) media, no mature *lipA* mRNA (921 bp) was detected from mycelium grown in either medium (Table 1). When the GMS or PMS growth media were supplemented with 0·5% soya bean oil or peanut oil, the mature *lipA* mRNAs (921 bp) were readily detected (Table 1; Fig. 2a,b). The genomic and cDNA sequences of *lipA* from the two *A. flavus* isolates, wool-1 and wool-2, were shown to be identical to that of *A. flavus* SRRC 1007 (data not shown). The expression pattern of *lipA* from these two isolates was also the same as that of *A. parasiticus* and *A. flavus* (Fig. 2a, b) in that in the presence of oil the mature mRNAs were readily detected. The CAM is oil rich and often used for aflatoxin production assays (Arseculeratne *et al.* 1969). When the fungal mycelia were grown in CAM, the *lipA* mature mRNAs from *A. parasiticus* SRRC 143 and *A. flavus* SRRC 1007 were detected (Fig. 2c). When the two nonaflatoxingenic *A. flavus* isolates, wool-1 and wool-2, were grown in the CAM, the *lipA* mature mRNAs were detected, but the level was significantly reduced based on the band intensity of RT-PCR amplification. The mature mRNAs detected from 72 h mycelia of the two *A. flavus* isolates, wool-1 and wool-2, were (estimated by visualization and by densitometry measurement) *ca* 20 and 60%, respectively, of the wild type strain *A. flavus* SRRC 1007 (Fig. 2c) grown under similar conditions.

Independent expressions of the *lipA* gene and aflatoxin pathway genes

Equivalent amounts of aflatoxins were produced in both GMS medium and GMS medium containing 0·5% soya bean oil (Fig. 3, lanes 1 and 2, and lanes 6 and 7).

Table 1 Expression of *lipA* (mature mRNA) and aflatoxin production in *Aspergillus* under different nutritional media conditions

Strains	Media*	*lipA* Mature mRNA detected (%)	Aflatoxins produced	Relative amount of aflatoxins (%)
A. parasiticus SRRC 143	GMS	Not detected	B_1,B_2,G_1,G_2	100
A. parasiticus SRRC 143	GMS + oil†	100	B_1,B_2,G_1,G_2	100
A. parasiticus SRRC 143	PMS	Not detected	No	0
A. parasiticus SRRC 143	PMS + oil	100	B_1,B_2,G_1,G_2	60
A. parasiticus SRRC 143	PMS (48 h) + oil (24 h)‡	50	B_1,B_2,G_1,G_2	20
A. parasiticus SRRC 143	Coconut	100	B_1,B_2,G_1,G_2	100
A. flavus SRRC 1007	GMS	Not detected	B_1,B_2	100
A. flavus SRRC 1007	GMS + oil	100	B_1,B_2	100
A. flavus SRRC 1007	PMS	Not detected	No	0
A. flavus SRRC 1007	PMS + oil	100	B_1,B_2	10
A. flavus SRRC 1007	Coconut	100	B_1,B_2	100
A. flavus isolate wool-1	GMS	Not detected	No	0
A. flavus isolate wool-1	GMS + oil	100	No	0
A. flavus isolate wool-1	PMS	Not detected	No	0
A. flavus isolate wool-1	PMS + oil	100	No	0
A. flavus isolate wool-1	Coconut	20	No	0
A. flavus isolate wool-2	GMS	Not detected	No	0
A. flavus isolate wool-2	GMS + oil	100	No	0
A. flavus isolate wool-2	PMS	Not detected	No	0
A. flavus isolate wool-2	PMS + oil	100	No	0
A. flavus isolate wool-2	Coconut	60	No	0
A. parasiticus CS10N2-disA	GMS	Not detected	No	0
A. parasiticus CS10N2-disA	GMS + oil	100	No	0
A. parasiticus CS10N2-disA	PMS	Not detected	No	0
A. parasiticus CS10N2-disA	PMS + oil	100	No	0
A. parasiticus CS10N2-disA	Coconut	100	No	0

*Fungal mycelia were grown in these media with or without 0·5% oil for 72 h except noted otherwise.

†Oil was added in the concentration of 0·5% to the media at the time of inoculation except noted below.

‡PMS medium for 48 h, then adding 0·5% oil and growing for additional 24 h.

Furthermore aflatoxin was detected in nonaflatoxin-inducive medium (PMS) when supplemented with 0·5% soya bean oil (Fig. 3, lanes 4 and 9). However, unlike aflatoxin pathway genes, the *lipA* gene is expressed and processed in the presence of peptone in the PMS medium as long as it was supplemented with 0·5% soya bean oil. The mature *lipA* mRNA was detected in both aflatoxin-inducive (GMS) and nonaflatoxin-inducive (PMS) media when supplemented with 0·5% soya bean oil. In the nonaflatoxigenic *A. flavus* isolates, wool-1 and wool-2, no aflatoxin was produced irrespective of medium utilized. When the two isolates were grown in either GMS or PMS medium containing 0·5% oil, the *lipA* gene was transcribed and mature mRNA was detected (Table 1).

The *aflR*, a positive regulatory gene, is responsible for the activation of the aflatoxin biosynthetic pathway genes (Chang *et al.* 1995). In order to investigate whether *aflR* exerts any influence on the expression of the *lipA* gene, production of mature mRNA by the *aflR*-disrupted mutant strain of *A. parasiticus* CS10N2-disA (Takahashi *et al.* 2002) was assessed. In this mutant, no aflatoxin pathway genes were transcribed and no aflatoxins were produced. RT-PCR of *lipA* gene, using primers which function effectively with *A. parasiticus* CS10N2-disA, demonstrated that the *lipA* gene was transcribed under all medium conditions used and that mature mRNA was detected only when 0·5% oil was supplemented in the growth media (Table 1). In other words, when the aflatoxin pathway regulatory gene, *aflR*, was disabled, *lipA* gene expression was not affected. No *aflR* binding motif (TCGxxxxxCGA; Ehrlich *et al.* 1999) was identified in the promoter region of the *lipA* gene sequence. Therefore, it is unlikely that the transcription of the *lipA* gene is under the regulation of *aflR*, as is the case with the majority, if not all, of the aflatoxin pathway genes (Chang

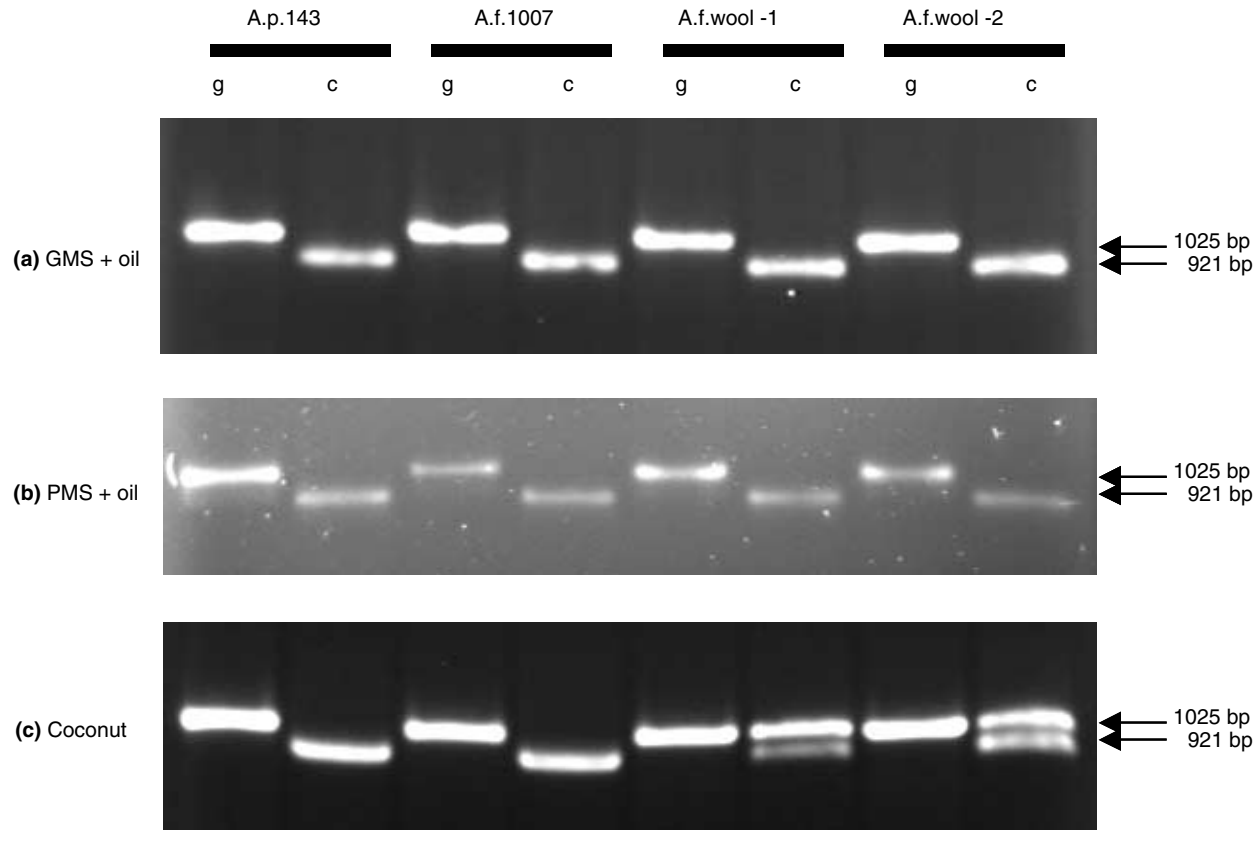

Fig. 2 Detection of the *lipA* transcript by RT-PCR. RT-PCR was performed to detect *lipA* mature mRNAs under different nutritional conditions from *A. parasiticus* SRRC 143, *A. flavus* SRRC 1007, and the two nonaflatoxigenic *A. flavus* isolates, wool-1 and wool-2, respectively. (a) Fungal mycelia grown in GMS medium supplemented with 0·5% soya bean oil. (b) Fungal mycelia grown in PMS medium supplemented with 0·5% soya bean oil. The lipase gene (*lipA*) was transcribed and mature mRNAs were detected (lanes c), but with less intensity compared with GMS medium supplemented with oil; and (c) fungal mycelia grown in coconut medium. The lipase gene (*lipA*) was transcribed and mature mRNAs were detected (lanes c). A.p. 143 represents PCR products amplified from *A. parasiticus* SRRC 143; A.f.1007 represents PCR products amplified from *A. flavus* SRRC 1007; A.f. wool-1 represents PCR products amplified from *A. flavus* isolate wool-1; A.f. wool-2 represents PCR products amplified from *A. flavus* isolate wool-2. Lane 'g' represents genomic PCR products amplified from genomic DNA purified from the respective fungal mycelia. Lane 'c' represents RT-PCR products amplified from first strand cDNA of respective fungal strain

et al. 1995; Ehrlich *et al.* 1999). Expression of *lipA* is induced by lipid, while aflatoxin-inducive or nonaflatoxin-inducive media alone did not support the expression of *lipA*. However, the aflatoxin pathway gene expression and eventually aflatoxin production is not totally dependent on lipid induction (Table 1; Fig. 3) as hexoses such as glucose and sucrose are known to be very good supporters of aflatoxin formation. The results demonstrated that lipid was required for the expression of *lipA*, but aflatoxin production did not necessarily require lipid. Lipases are substrate-induced enzymes. The metabolite 'acetate' may be produced during hydrolysis of lipids by lipases. Acetate is believed to be the earliest aflatoxin precursor. The aflatoxin production in response to lipase gene expression might be indirectly related to toxin synthesis through providing the building block 'acetate' for aflatoxin biosynthesis. It seems, therefore, that the expression of *lipA* gene has no direct relationship with the expression of aflatoxin pathway genes (*aflR*) and so no direct relationship exists between lipid metabolism and aflatoxin formation.

Lipid induces, supports or promotes aflatoxin production

Aflatoxigenic fungal strains grown in the different media (GMS, PMS and each supplemented with 0·5% oil) were assessed for the level of aflatoxin production. The amount of aflatoxin produced in GMS medium was treated as 100% (Table 1; Fig. 3, lanes 1 and 6). When the fungus was grown in the GMS medium supplemented with 0·5% soya bean oil, no noticeable increase in aflatoxin production was detected (Table 1; Fig. 3, lanes 2 and 7). When the fungus was grown

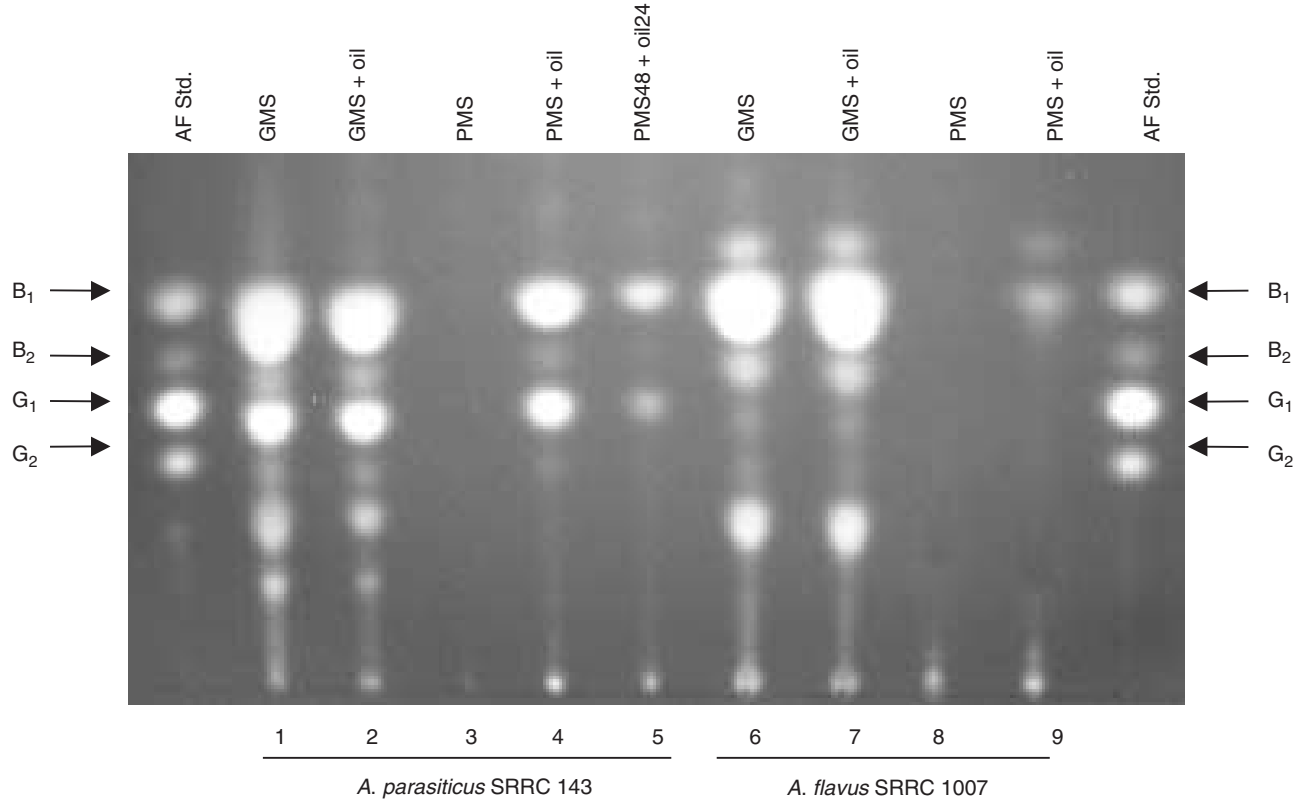

Fig. 3 Thin-layer chromatographic (TLC) assay of aflatoxin production in different media conditions. Aflatoxins were extracted from a mixture of 72 h mycelia and the growth medium. The extracted aflatoxins were loaded onto TLC plates. TLC plates were developed in an ether : methanol : water (96 : 3 : 1, v/v/v) solvent system. A total of 20 μg of known compounds of aflatoxin (B_1, B_2, G_1 and G_2) standards (AF Std) were loaded onto the TLC plates for quantitating aflatoxins produced in the various media conditions. Lanes 1–5 are *A. parasiticus* SRRC 143 and lanes 6–9 are *A. flavus* SRRC 1007. Lanes 1 and 6 are GMS, lanes 2 and 7 are GMS supplemented with 0·5% soya bean oil, lanes 3 and 8 are PMS, and lanes 4 and 9 are PMS supplemented with 0·5% soya bean oil. Lane 5 is *A. parasiticus* SRRC 143 grown in PMS for 48 h and then adding 0·5% soya bean oil and continuing growth for additional 24 h

in the PMS medium, no aflatoxin was detected (Table 1; Fig. 3, lanes 3 and 8). When this PMS medium was supplemented with 0·5% soya bean oil, marked aflatoxin production was experienced (Fig. 3, lane 4, Fig. 3, lane 9) albeit not as great as supported by GMS medium. The results demonstrated that the fungus may use oil as carbon source and that lipid may also promote the expression of aflatoxin pathway genes and support aflatoxin formation albeit at low efficiency compared with glucose or sucrose rich media.

Fungal mycelia from PMS medium (nonaflatoxin-inducive condition) supplemented with 0·5% oil, produced aflatoxins within 24 h (Fig. 3, lane 5). The whole process of signal transduction involves many genetic and biochemical steps. At a minimum consisting of lipid-induced *lipA* gene transcription, transcript processing, lipid degradation, induction of aflatoxin pathway gene expression and finally aflatoxin biosynthesis. Approximately 5–10% of aflatoxins (Fig. 3, lane 5) were accumulated within a 24-h culture compared with a 72-h culture grown in the aflatoxin-inducive media (Fig. 3, lane 1 or 2). When the fungal mycelia is grown in the PMS medium supplemented with 0·5% oil for 72 h (Fig. 3, lane 4), the amount of aflatoxins produced is about 60% of that produced by growing in the GMS medium for the same duration (Fig. 3, lane 1 or 2). In contrast fungal mycelia grown in the GMS medium, and GMS supplemented with 0·5% oil, revealed no significant difference in the amount of aflatoxin production (Fig. 3, lanes 1 and 2, lanes 6 and 7) between species and supplementation or lack of supplementation.

DISCUSSION

The first AA residue of mature lipase enzyme in the closely related species *A. oryzae* and also in the nonrelated species *P. camembertii* was reported to be an aspartic acid (Tsuchiya *et al.* 1996, accession no. D85895; Yamaguchi *et al.* 1991, accession no. D90315). This aspartic acid reside at position

29 (Fig. 1 printed in boldface letter and underlined) was identified as N-terminal residue of putative mature lipase in *A. parasiticus* and *A. flavus*. Excluding the 28 AA of leader sequence, the putative mature enzyme should consist of 278 AA with a calculated molecular mass of 30·4 kDa. It was reported (Toida *et al.* 1995) that the purified lipase protein (L2) from *A. oryzae* was between 39 and 41 kDa. It is possible that the molecular weight discrepancy occuring between purified native mature enzyme and the deduced molecular mass was due to glycosylation of the enzyme. A consensus N-glycosylation site (NTTV) was also identified in this lipase from *A. parasiticus* and *A. flavus* to be at position 252 (Fig. 1; Yamaguchi *et al.* 1991; Tsuchiya *et al.* 1996). The conserved AA sequence motif (GXSXG) at position 171–175 and a catalytic triad consisting of serine (S-173), aspartic acid (D-226) and histidine (H-288) for lipolytic enzymes were identified as well (Fig. 1; Yamaguchi *et al.* 1991; Tsuchiya *et al.* 1996).

BLAST search against the NCBI (http://www.ncbi.nlm.nih.gov) GenBank database and *A. nidulans* genome database (http://www-genome.wi.mit.edu/annotation/fungi/aspergillus/) for the cloned *lipA* gene sequence demonstrated that no significant sequence homology has been identified in the database at nucleotide and AA levels. However, we identified an *A. nidulans lipA* gene (accession no. AF424740) encoding for triacylglycerol lipase in the NCBI database with no sequence homology between the *lipA* in *A. parasiticus*, *A. flavus* and *A. nidulans*. The *lipA* gene in *A. nidulans* is probably doing the same function as our cloned example.

It was demonstrated that supplementation of lipid (0·5% oil) in the nonaflatoxin-conducive medium (PMS) promoted aflatoxin biosynthesis (Fig. 3, lanes 5 and 9). Aflatoxin biosynthesis involves over 20 aflatoxin pathway genes and their encoded enzymes. We postulate that the function of the lipids could be either as a carbon source or as an inducer for aflatoxin production. However, unlike sucrose or glucose (GMS medium), the lipids are not the preferred carbon source for aflatoxin production. These data do suggest that lipase or specific lipase actions involved in lipid metabolism may be important in promoting aflatoxin formation, possibly during fungal invasion of lipid-rich environments (such as seeds of corn, cotton and peanuts). The cloning and expression of lipase gene may further help our understanding of fungal invasion, the complex regulation of aflatoxin biosynthesis, and the potential existence of signal transduction between primary and secondary metabolism.

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REFERENCES

Adye, J. and Mateles, R.I. (1964) Incorporation of labeled compounds into aflatoxins. *Biochimica Biophysica Acta* **86**, 418–420.

Arseculeratne, S.N., De Silva, L.M., Wijesundera, S. and Bandunatha, C.H.S.R. (1969) Coconut as a medium for the experimental production of aflatoxin. *Applied Microbiology* **18**, 88–94.

Aziz, N.H., El-Zeany, S.A. and Moussa, L.A.A. (2002) Influence of gamma-irradiation and maize lipids on the production of aflatoxin B-1 by *Aspergillus flavus*. *NAHRUNG-FOOD* **46**, 327–331.

Bhatnagar, D., Ehrlich, K.C. and Cleveland, T.E. (1992) Oxidation-reduction reactions in biosynthesis of secondary metabolites. In *Handbook of Applied Mycology, Mycotoxins in Ecological Systems*, vol. 5. ed. Bhatnagar, D., Lillehoj, E.B. and Arora, D.K. pp. 255–286. New York, NY: Marcel Dekker, Inc.

Bhatnagar, D., Yu, J. and Ehrlich, K.C. (2002) Toxins of filamentous fungi. In *Fungal Allergy and Pathogenicity* ed. Breitenbach, M., Crameri, R. and Lehrer, S.B. Basel: Karger (Book chapter Review).*Chemical Immunology* **81**, pp. 167–206.

Brown, D.W., Yu, J.H., Kelkar, H.S., Fernandes, M., Nesbitt, T.C., Keller, N.P., Adams, T.H. and Leonard, T.J. (1996) Twenty-five coregulated transcripts define a sterigmatocystin gene cluster in *Aspergillus nidulans*. *Proceedings of National Academy of Sciences USA* **93**, 1418–1422.

Chang, P.-K. and Yu, J. (2002) Characterization of a partial duplication of the aflatoxin gene cluster in *Aspergillus parasiticus* ATCC 56775. *Applied Microbiology and Biotechnology* **58**, 632–636.

Chang, P.-K., Cary, J.W., Bhatnagar, D., Cleveland, T.E., Bennett, J.W., Linz, J.E., Woloshuk, C.P. and Payne, G.A. (1993) Cloning of the *Aspergillus parasiticus apa-2* gene associated with the regulation of aflatoxin biosynthesis. *Applied and Environmental Microbiology* **59**, 3273–3279.

Chang, P.-K., Ehrlich, K.C., Yu, J., Bhatnagar, D. and Cleveland, T.E. (1995) Increased expression of *Aspergillus parasiticus aflR*, encoding a sequence specific DNA-binding protein, relieves nitrate inhibition of aflatoxin biosynthesis. *Applied and Environmental Microbiology* **61**, 2372–2377.

Chang, P.-K., Yu, J., Bhatnagar, D. and Cleveland, T.E. (2000) Characterization of the *Aspergillus parasiticus* major nitrogen regulatory gene, *areA*. *Biochimica et Biophysica Acta* **1491**, 263–266.

Cleveland, T.E. and Bhatnagar, D. (1992) Molecular strategies for reducing aflatoxin levels in crops before harvest. In *Molecular Approaches to Improving Food Quality and Safety* ed. Bhatnagar, D. and Cleveland, T.E. pp. 205–228. New York, NY: Van Nostrand Reinhold.

Cleveland, T.E., Cary, J.W., Brown, R.L., Bhatnagar, D., Yu, J., Chang, P.-K., Chlan, C.A. and Rajasekaran, K. (1997) Use of biotechnology to eliminate aflatoxin in preharvest crops. *Bulletin of Institute Comprehensive Agricultural Sciences, Kinki University* **5**, 75–90.

Davis, N.D., Iyer, S.K. and Diener, U.L. (1987) Improved method of screening for aflatoxin with a coconut agar medium. *Applied Environmental Microbiology* **53**, 1593–1595.

De Luca, C., Passi, S., Fabbri, A.A. and Fanelli, C. (1995) Ergosterol oxidation may be considered a signal for fungal growth and aflatoxin production in *Aspergillus parasiticus*. *Food Additive Contamination* **12**, 445–450.

Dutton, M.F. (1988) Enzymes and aflatoxin biosynthesis. *Microbiology Review* **52**, 274–295.

Dyer, S.K. and McCammon, S. (1994) Detection of toxigenic isolates of *Aspergillus flavus* and related species on coconut cream agar. *Journal of Applied Bacteriology* **76**, 75–78.

Ehrlich, K.C., Montalbano, B.G. and Cary, J.W. (1999) Binding of the C6-zinc cluster protein, AFLR, to the promoter of aflatoxin pathway biosynthesis genes in *Aspergillus parasiticus*. *Gene* **230**, 249–257.

Fanelli, C. and Fabbri, A.A. (1989) Relationship between lipids and aflatoxin biosynthesis. *Mycopathologia* **107**, 115–120.

Fanelli, C., Fabbri, A.A., Finotti, E. and Passi, S. (1983) Stimulation of aflatoxin biosynthesis by lipophilic epoxides. *Journal of General Microbiology* **129**, 1721–1723.

Fanelli, C., Fabbri, A.A., Brasini, S., De Luca, C. and Passi, S. (1995) Effect of different inhibitors of sterol biosynthesis on both fungal growth and aflatoxin production. *Natural Toxins* **3**, 109–113.

Jelinek, C.F., Pohland, A.E. and Wood, G.E. (1989) Worldwide occurrence of mycotoxins in foods and feeds – an update. *Journal of Association Official Analytical Chemistry* **72**, 223–230.

Meyers, D.M., Obrian, G., Du, W.L., Bhatnagar, D. and Payne, G.A. (1998) Characterization of *aflJ*, a gene required for conversion of pathway intermediates to aflatoxin. *Applied and Environmental Microbiology* **64**, 3713–3717.

Ohnishi, K. (1982a) Change of lipids during miso-fermentation. *Nippon Jozo Kyokaishi* **78**, 848–853 (in Japanese).

Ohnishi, K. (1982b) Effect of lipase activity on aging and quality of miso (Study on lipids of miso during aging, Part I). *Nippon Shokuhin Kogyo Gakkaishi* **29**, 85–92 (in Japanese).

Payne, G.A. and Brown, M.P. (1998) Genetics and physiology of aflatoxin biosynthesis. *Annual Review of Phytopathology* **36**, 329–362.

Payne, G.A., Nystrom, G.J., Bhatnagar, D., Cleveland, T.E. and Woloshuk, C.P. (1993) Cloning of the *afl-2* gene involved in aflatoxin biosynthesis from *Aspergillus flavus*. *Applied and Environmental Microbiology* **59**, 156–162.

Takahashi, T., Chang, P.-K., Matsushima, K., Yu, J., Abe, K., Bhatnagar, D., Cleveland, T.E. and Koyama, Y. (2002) Non-functionality of *Aspergillus sojae aflR* in a strain of *Aspergillus parasiticus* with a disrupted *aflR* gene. *Applied and Environmental Microbiology* **68**, 3737–3743.

Toida, J., Kondoh, K., Fukuzawa, M., Ohnishi, K. and Sekiguchi, J. (1995) Purification and characterization of a lipase from *Aspergillus oryzae*. *Bioscience Biotechnology and Biochemistry* **59**, 1199–1203.

Trail, F., Mahanti, N., Rarick, M., Mehigh, R., Liang, S.-H., Zhou, R. and Linz, J.E. (1995) A physical and transcriptional map of an aflatoxin gene cluster in *Aspergillus parasiticus* and the functional disruption of a gene involved early in the aflatoxin pathway. *Applied and Environmental Microbiology* **61**, 2665–2673.

Tsuchiya, A., Nakazawa, H., Toida, J., Ohnishi, K. and Sekiguchi, J. (1996) Cloning and nucleotide sequence of mono- and diacylglycerol lipase gene (*mdlB*) of *Aspergillus oryzae*. *FEMS Microbiology Letter* **143**, 63–67.

Woloshuk, C.P. and Prieto, R. (1998) Genetic organization and function of aflatoxin B_1 biosynthetic genes. *FEMS Microbiology Letter* **160**, 169–176.

Woloshuk, C.P., Cavaletto, J.R. and Cleveland, T.E. (1997) Inducers of aflatoxin biosynthesis from colonized maize kernels are generated by an amylase activity from *Aspergillus flavus*. *Phytopathology* **87**, 164–169.

Yabe, K., Ando, Y., Hashimoto, J. and Hamasaki, T. (1989) Two distinct *O*-methyltransferases in aflatoxin biosynthesis. *Applied and Environmental Microbiology* **55**, 2172–2177.

Yabe, K., Matsushima, K., Koyama, T. and Hamasaki, T. (1998) Purification and characterization of *O*-methyltransferase I involved in conversion of demethylsterigmatocystin to sterigmatocystin and of dihydrodemethylsterigmatocystin to dihydrosterigmatocystin during aflatoxin biosynthesis. *Applied and Environmental Microbiology* **64**, 166–171.

Yamaguchi, S., Mase, T. and Takeuchi, K. (1991) Cloning and structure of the mono-and diacylglycerol lipase-encoding gene from *Penicillium cumembertii* U-150. *Gene* **103**, 61–67.

Yu, J., Cary, J.W., Bhatnagar, D., Cleveland, T.E., Keller, N.P. and Chu, F.S. (1993) Cloning and characterization of a cDNA from *Aspergillus parasiticus* encoding an *O*-methyltransferase involved in aflatoxin biosynthesis. *Applied and Environmental Microbiology* **59**, 3564–3571.

Yu, J., Chang, P.-K., Cary, J.W., Wright, M., Bhatnagar, D., Cleveland, T.E., Payne, G.A. and Linz, J.E. (1995) Comparative mapping of aflatoxin pathway gene clusters in *Aspergillus parasiticus* and *Aspergillus flavus*. *Applied and Environmental Microbiology* **61**, 2365–2371.

Yu, J., Chang, P.-K., Ehrlich, K.C., Cary, J.W., Montalbano, B.G., Dyer, J.M., Bhatnagar, D. and Cleveland, T.E. (1998) Characterization of the critical amino acids of an *Aspergillus parasiticus* cytochrome P-450 monooxygenase encoded by *ordA* involved in aflatoxin B_1, G_1, B_2, and G_2 biosynthesis. *Applied and Environmental Microbiology* **64**, 4834–4841.

Yu, J., Chang, P.-K., Bhatnagar, D. and Cleveland, T.E. (2000a) Genes encoding cytochrome P450 and monooxygenase enzymes define one end of the aflatoxin pathway gene cluster in *Aspergillus parasiticus*. *Applied Microbiology and Biotechnology* **53**, 583–590.

Yu, J., Woloshuk, C.P., Bhatnagar, D. and Cleveland, T.E. (2000b) Cloning and characterization of *avfA* and *omtB* genes involved in aflatoxin biosynthesis in three *Aspergillus* species. *Gene* **248**, 157–167.

Yu, J., Chang, P.-K., Bhatnagar, D. and Cleveland, T.E. (2000c) Cloning of a sugar utilization gene cluster in *Aspergillus parasiticus*. *Biochimica et Biophysica Acta* **1493**, 211–214.